*Parasite Immunology*, 2006, **28**, 315–327 DOI: 10.1111/j.1365-3024.2006.00849.x

**Review Article**

# Prospects for recombinant vaccines against *Babesia bovis* and related parasites

W. C. BROWN,[1] J. NORIMINE,[1] W. L. GOFF,[2] C. E. SUAREZ[2] & T. F. MCELWAIN[1]

[1]Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA, USA and [2]Animal Disease Research Unit, United States Department of Agriculture, Agricultural Research Service, Washington State University, Pullman, WA, USA

## SUMMARY

*Babesial parasites infect cattle in tropical and temperate regions of the world and cause significant morbidity and mortality. Discovery of protective antigens that could be used in a killed vaccine has been slow and to date there are few promising vaccine candidates for cattle* Babesia. *This review describes mechanisms of protective innate and adaptive immune responses to babesial parasites and different strategies to identify potentially protective protein antigens of* B. bovis, B. bigemina, *and* B. divergens. *Successful parasites often cause persistent infection, and this paper also discusses how* B. bovis *evades and regulates the immune response to promote survival of parasite and host. Development of successful non-living recombinant vaccines will depend on increased understanding of protective immune mechanisms and availability of parasite genomes.*

***Keywords*** Babesia bigemina, Babesia bovis, Babesia divergens, *babesiosis, cattle, vaccine antigens*

*Correspondence*: Dr Wendy C. Brown, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040, USA (e-mail: wbrown@vetmed.wsu.edu).
*Received: 13 October 2005*
*Accepted for publication: 30 January 2006*

## INTRODUCTION

Babesiosis in cattle, which has also commonly been called tick fever, redwater, and piroplasmosis, is caused by several different species of *Babesia* found throughout tropical and temperate areas of the world. The three most important pathogens are *Babesia bovis, B. bigemina*, and *B. divergens.* Although these organisms are phylogenetically closely related (1,2), and, in the case of *B. bovis* and *B. bigemina*, transmitted by the same tick vector *Rhipicephalus (Boophilus) microplus*, the diseases caused by these parasites are remarkably different (3). *Babesia bovis* is the most pathogenic organism, resulting in high mortality rates among susceptible cattle. The disease caused by *B. bovis* is characterized by fever, anaemia, anorexia, cachexia, haemoglobinurea and a hypotensive shock syndrome. In acutely ill animals, parasitized erythrocytes are sequestered in the microcapillary beds of the brain and lung, resulting in low levels of parasitaemia in the periphery, cerebral babesiosis, and a respiratory distress syndrome associated with neutrophil infiltration into the lung capillaries, vascular permeability, and oedema (reviewed in (4)). The severe pathogenesis is thought to be partially immune-mediated, and overproduction of soluble mediators including IFN-γ, TNF-α, and nitric oxide (NO) that are associated with protective immunity against many intracellular pathogens has been implicated (4,5). In support of this, Hemmer *et al*. (6) demonstrated that TNF-α played a significant role in the pathogenesis of virulent WA-1 *Babesia* infection.

*Babesia bigemina* and *B. divergens* present with less severe pathology, although disease can vary from mild to severe anaemia, and can be fatal. The course of disease with all of these parasites is in part determined by age and immune status of the host, and differing virulence among parasite strains (3,7).

Recovery from infection with *Babesia,* either naturally or following chemotherapeutic intervention, generally results in parasite persistence and protection against disease following reinfection. This concomitant immunity may contribute to a protective immune response by providing a continual source of antigenic stimulation, which in one model of *Leishmania major* infection, is required for the maintenance of effector memory T cell responses (8). Concomitant immunity is the basis for using live *Babesia* vaccines attenuated by passage through splenectomized cattle (9). However, live vaccines are not without risk, which includes transmission of other blood-borne pathogens and failure due to vaccine breakthroughs (9).

Immunity following immunization with live, attenuated *B. bovis* vaccine is known to last after parasites have been eliminated by drug treatment (10). Similar conclusions were reached with *B. divergens* infection (11). However, the long-term duration of such 'sterile' immunity is really unknown. The ability to confer some level of protective immunity following immunization with killed parasites or parasite extracts upon homologous or heterologous strain challenge has provided a rationale for developing subunit vaccines (12–14).

This review article examines current understanding of protective innate and adaptive immune mechanisms to cattle *Babesia*, strategies for developing nonliving vaccines and identification of immunogenic proteins that have been considered as candidates for inclusion in a subunit vaccine, and challenges imposed by antigenic variation of surface proteins.

## MECHANSIMS OF PROTECTIVE IMMUNITY

The mechanisms of immunity to babesial parasites are hypothesized to require innate as well as adaptive responses. Several reviews have described a model of protective immune mechanisms for *B. bovis* (15–17). Most of our knowledge of protective adaptive immune responses to bovine babesial pathogens has been deduced from mouse models of infection.

### Mouse models of immunity to *Babesia*

Considerable research has been done using murine models of *Babesia* that infect humans, notably *B. microti* and the virulent WA1 *Babesia* (reviewed in (18)). Although these parasites are phylogenetically more related to *Theileria* than to cattle *Babesia* (1,2), they are relevant because they cause disease similar to that caused by bovine babesial parasites. In *B. microti*-infected mice, many studies have clearly shown that $CD4^+$ T cells, but not $CD8^+$ T cells, are required for protective immunity (19–21). The requirement for IFN-γ is less clear. One study using IFN-γ knockout mice showed delayed but eventual resolution of disease caused by *B. microti* (21), whereas a separate study showed no protection against challenge with this parasite in mice depleted of IFN-γ by specific antibody treatment (20). Differences may be related to the use of different parasite strains. Several studies have also shown that antibody does not protect mice against *B. microti* infection (20–22). Nevertheless, *in vitro* inhibition of parasite growth has been observed with *B. microti*-immune serum (23). These results show that *in vitro* parasite inhibition assays do not necessarily predict the protective efficacy of antibody *in vivo*.

*Babesia microti* is a relatively benign pathogen in mice, and a more virulent *Babesia* parasite, designated WA1 for its origin in a human patient from Washington State (24), may be a more relevant mouse model for the virulent *B. bovis* pathogen. In contrast to the self-limiting *B. microti* infection in C3H/HeN mice, WA1 *Babesia* infection caused severe clinical disease by 10 days and 100% mortality (6). The WA1 *Babesia* infection was characterized by production of inflammatory cytokines TNF-α by γδ T cells and IFN-γ by $CD8^+$ T cells. The infection was less severe in TNF receptor-deficient mice, which had 90% survival rates (6). A subsequent study with gene knockout mice extended these observations and showed a protective role for IL-12, IFN-γ, and NO in acute WA1 *Babesia* infection (25). Neither B cells nor $CD4^+$ T cells appeared to contribute to immunity, whereas NK cells and macrophages were important. These data suggest that for the acutely pathogenic WA1 *Babesia*, innate immune mechanisms involving IL-12- and IFN-γ-mediated macrophage activation and NO production are critical to controlling infection so that acquired immunity can develop.

### Innate immune responses to cattle *Babesia*

It has been proposed that resolution of an acute infection in naïve calves infected with virulent *B. bovis* parasites depends on a strong innate immune response in the spleen that causes activation of macrophages via IFN-γ and parasite-derived products, and results in killing of the organisms by phagocytosis and production of toxic macrophage metabolites, including NO. In support of this, a study comparing protected and unprotected cattle following vaccination with a recombinant antigen, 11C5, showed that resolution of infection correlated positively with *ex vivo* production of TNF-α by peripheral blood mononuclear cells and a spike in IFN-γ production (26). The importance of the spleen in controlling infection is documented, as splenectomy results in recrudescence of parasitaemia and clinical disease in persistently infected cattle (3).


 

Interestingly, young calves less than 6 months of age are relatively resistant to developing the severe form of disease typically observed in susceptible adults upon initial infection with *B. bovis* (27,28). Calves under 1 year of age are similarly resistant to disease caused by *B. divergens* (29). This age-related resistance is not due only to the protective effects of maternal antibody, because passively transferred antibody has a life-span less than the duration of resistance, and calves born from serologically negative dams or in disease-free regions, that were subsequently infected with the parasite, were also resistant (28,30). The resistance of young animals compared with adults to *B. bovis* infection has been shown to reflect an earlier expression in calves in the spleen of IL-12, IFN-γ and NO and increased numbers of NK-like cells following challenge with the virulent $T_2$Bo isolate (28,31). Previous studies had documented that *B. bovis* induced NO production in macrophages in an IFN-γ-dependent manner, and demonstrated *in vitro* killing of *B. bovis* by NO (32–34). In young calves, splenic NK cells produce IFN-γ in response to IL-18 and IL-12 or supernatants from *B. bovis*-stimulated macrophages that contain IL-12 (35). Collectively, these data argue that the age-related resistance to *B. bovis* is in part mediated by an early IL-12- and NK cell-mediated IFN-γ response, which activates macrophages to produce sufficient NO to kill the parasites early enough to prevent the pathological consequences of infection, so that adaptive immune responses can ensue. The parasite molecules that stimulate innate immunity include CpG DNA and lipids, reviewed in detail elsewhere (17,36), are discussed below.

To be successful, a parasite should not kill its host but rather coexist in a state of persistent infection. To this end, many pathogens may have adapted strategies to stimulate an innate immune response that controls infection levels, but that is incapable of completely eliminating the infection. Different components of various pathogens, including bacteria, fungi, viruses, and parasites stimulate innate immune responses by binding to mammalian Toll-like receptors (TLRs) (37–41). TLRs expressed on a variety of leucocyte subsets recognize conserved molecular motifs called pathogen-associated molecular patterns (PAMPs) and TLR engagement initiates the innate immune response to many pathogens, resulting in cytokine and costimulatory molecule expression by antigen-presenting cells required for initiation of adaptive immune responses. Examples of PAMPs and their respective TLRs include lipopeptide (TLR2), single and double stranded viral RNA (TLRs3 and 4), bacterial lipopolysaccharide (TLR4), flagellin (TLR5), poly G motifs of DNA (TLR8), non-methylated CpG motifs of DNA (TLR9), and profilin (TLR11). We had observed that *B. bovis* merozoite extracts non-specifically activated proliferation of bovine PBMC. Subsequent studies determined that this response was in part due to the presence of non-methylated CpG motifs in *B. bovis* DNA that activated proliferation of B cells (42). We went on to show that merozoite extracts, *B. bovis* lipid, and purified DNA from *B. bovis, Trypanosoma brucei*, and *T. cruzi* as well as *E. coli*, activated macrophages to produce IL-1β, IL-12, TNF-α and NO (34,43). TLR9 is expressed in cattle (44), and these studies demonstrated that it is functional.

Profilin, recently identified in *Toxoplasma gondii* and other protozoa, is an actin-binding protein that induced production of large amounts of IL-12 from murine dendritic cells in a TLR11-dependent manner (41). By blasting the *B. bovis* Texas $T_2$Bo strain genomic sequence for homologous genes, we identified a 17·5-kDa profilin-like protein that had 34–64% identity to *Plasmodium falciparum, Toxoplasma gondii*, and *Theileria parva* profilins. However, neither *Cryptosporidium parvum* profilin (kindly provided to us by A. Sher, Laboratory of Parasitic Diseases, NIH) nor recombinant *B. bovis* profilin stimulated IL-12 production by bovine APC, under the same experimental conditions where *B. bovis* DNA and *E. coli* DNA did (Norimine, Suarez, and Brown, unpublished observations; 43). We have not identified homologues for TLR11 to date in the available bovine genome, which probably explains our results. Interestingly, TLR11 is not expressed by human cells (45) nor by several other mammalian species, including cat, dog and chimpanzee (46). Thus, the role of apicomplexan profilins in non-murine mammalian immunity to infection by protozoa is currently unknown.

## Adaptive immunity to cattle *Babesia*

The ability to precisely define the adaptive immune responses in cattle that result in protective immunity to babesial parasites is limited by the large animal model, where knocking out specific lymphocyte subsets or performing adoptive transfer studies with defined cell populations is problematic. A brief summary of what is known regarding protective mechanisms and *in vitro* correlates of protective immunity is provided.

Because cattle *Babesia* parasites infect only erythrocytes, the adaptive immune response to subsequent infection and protection against clinical disease is dependent on presentation of parasite antigens to $CD4^+$ T-lymphocytes by professional antigen-presenting cells. Control of infection is probably mediated through destruction of infected erythrocytes by activated splenic macrophages and by neutralizing antibodies directed against extracellular merozoites and the infected erythrocyte surface-variable erythrocyte surface antigens, defined as VESA1 in *B. bovis* (47). Both of these immune mechanisms depend on $CD4^+$ T cells. The role of protective antibody in *B. bovis* infection was demonstrated



Journal compilation © 2006 Blackwell Publishing Ltd, *Parasite Immunology*, **28**, 315–327

by passive administration of immune serum or a mixture of IgG1 and IgG2 that provided protection against homologous strain challenge (13).

Characterizing the antigen-specific T cell response in cattle protected from *Babesia* infection following infection and treatment has similarly revealed that lymphocytes that respond *in vitro* to *B. bovis* and *B. bigemina* antigens are $CD4^+$ T cells (48,49). At the clonal level, all $CD4^+$ T cells specific for *B. bovis* express IFN-γ and some also express IL-4 mRNA (50). When assayed for helper T cell function *ex vivo*, *B. bigemina*-specific $CD4^+$ T cells that expressed relatively high levels of IFN-γ vs. IL-4 enhanced IgG1 and IgG2 secretion by autologous B-lymphocytes in co-culture with specific *Babesia* antigen (51). This result demonstrates a functional role of T helper cell IFN-γ for isotype switching to IgG2 (52), the best opsonizing antibody isotype in cattle (53).

Understanding the innate mechanisms of resistance in young animals to acute *B. bovis* infection, and understanding acquired immune mechanisms that result in continual control of parasitaemia to persistent levels in adult cattle that survive infection, are important for devising strategies to induce a protective immune response by vaccination. Knowledge of innate and resulting adaptive immune mechanisms of protection will help direct the use of adjuvants to stimulate the appropriate responses.

## STRATEGIES TO IDENTIFY PROTECTIVE ANTIGENS

The following section will review strategies that have been used to identify potentially protective *Babesia* protein antigens and the limitations of these strategies. It is hoped that some of the limitations can be overcome once the complete genome sequences for *B. bovis, B. bigemina* and *B. divergens* are obtained. Much of our knowledge of immunogenic babesial proteins is based on their immunodominance. These proteins have been identified by hyperimmune bovine serum or mouse monoclonal antibodies. A combined genomic and proteomic approach to identify novel vaccine antigens will also enable the identification of subdominant antigens; those antigens against which little or no immune response is made during infection. Subdominant antigens may prove to be more effective as vaccine candidates than immunodominant antigens, as demonstrated for certain viral proteins (54,55). It seems intuitive that for a parasite to survive, expression of immunodominant antigens that would be targeted by a protective immune response would be lethal for the parasite. Yet babesial parasites persist in the face of strong immune responses. Therefore, more rational choices of vaccine antigens might be those that do not naturally evoke a strong immune response. This idea was proposed by Byron Waksman in the 1970s and is referred to as the 'Waksman Postulate' (56). A combined genomic and proteomic approach to antigen discovery which can lead to identifying subdominant antigens, combined with an ability to enumerate and track $CD4^+$ T lymphocytes *in vivo* following immunization or challenge, will facilitate a better understanding of the reasons behind vaccine success or failure. The antigens described in the following sections are listed in Table 1.

### Empirical approach to identifying vaccine antigens

Merozoite proteins or culture supernatants (exoantigens) have been fractionated in a variety of ways and individual fractions tested for induction of protective immunity in cattle or animal models (reviewed in (16,57)). As summarized by Wright *et al*. (12) five secreted *B. bovis* merozoite antigens were identified that were neither serologically immunodominant nor particularly abundant. Four of these proteins were defined. One is a 77–80-kDa protein (Bv80) also called Bb-1 (58) and now designated spherical body protein 1 (SBP1) because of its localization to the spherical body organelles (59). This protein was originally identified as a component of a dextran sulphate-precipitated antigen, that when used to immunize cattle, was partially protective (60). The other three proteins were a 38-kDa cysteine-rich protein designated 12D3, a 60kDa rhoptry protein designated T21B4 and also called Bv60 and rhoptry-associated protein 1 (RAP-1) (61), and a high molecular weight antigen designated 11C5. 12D3 localized to the apical end of the merozoite and the cytoplasm of infected erythrocytes, suggesting that like RAP-1, it is a secreted protein (62). Later studies showed that 12D3 contains several T cell epitopes recognized by 12D3-immunized cattle (63), although our laboratory was consistently unable to stimulate recall $CD4^+$ T cell responses with recombinant 12D3, using lymphocytes obtained from infected and recovered cattle that otherwise responded to *B. bovis* and specific antigens, such as RAP-1 (Brown, unpublished observations). A truncated form of recombinant RAP-1 fused to GST was reported to confer partial protection against challenge, determined as reduction in packed erythrocyte volumes (12). Recombinant proteins consisting of glutathione-S-transferase (GST)-12D3 and GST-11C5 used alone or in combination with either Quil A saponin or complete Freund's adjuvant were also able to induce partial protection, but the greatest protection was achieved when more than one protein was given (12). A more recent study found that immunization with recombinant 11C5 protein resulted in control of parasitaemia in only 9 of 15 cattle (26).

Some protective immunity against homologous strain challenge was also observed when culture supernatants of


 

**Table 1** Candidate vaccine antigens of *Babesia bovis*, *Babesia bigemina*, and *Babesia divergens*

| Parasite antigen | Approach used[a] | Immunological response to individual proteins | | |
|---|---|---|---|---|
| | | Memory T cell response[b] | *In vitro* Ab neutralization[c] | *In vivo* protection[d] |
| *B. bovis* | | | | |
| 77–80-kDa SBP1 | Empirical, antibody, T cells | +[e] | ND[f] | ND |
| 60-kDa RAP-1 | Empirical, antibody, T cells | + | + | +/–[e] |
| 38-kDa 12D3 | Empirical, T cells | –[e] | ND | + |
| High mw 11C5 | Empirical | ND | ND | + |
| 42-kDa MSA-1 | Antibody, T cells | + | + | – |
| 44/48-kDa MSA-2a1/a2 | Antibody | – | + | ND |
| 35-kDa MSA-2b | Genomic | – | + | ND |
| 30-kDa MSA-2c | Genomic | – | + | ND |
| 225-kDa SBP2 | Antibody | ND | ND | ND |
| 135-kDa SBP3 | Antibody | ND | ND | ND |
| VESA1 | Antibody | ND | ND | ND |
| 78-kDa ACS1[g] | T cells | + | ND | ND |
| 34-kDa P0[g] | T cells | – | ND | ND |
| 82-kDa AMA-1 | Genomic | ND | + | ND |
| 75-kDa TRAP | Genomic | ND | + | ND |
| 18-kDa profilin[g] | Genomic | – | ND | ND |
| *B. bigemina* | | | | |
| 58 kDa RAP-1a | Antibody | + | ND | + |
| gp45 | Antibody | ND | ND | + |
| gp55 | Antibody | ND | ND | + |
| *B. divergens* | | | | |
| Bd37 | Empirical | ND | + | + (Gerbils) |

[a]The approach used to identify the antigens was an empirical approach, detection by bovine immune sera or monoclonal antibodies, stimulation of memory $CD4^+$ T cells from immune cattle, or a genomic approach. [b]Recombinant or native proteins were tested in T cell proliferation assays using T cells from cattle recovered from infection. [c]Merozoite neutralization assays were performed with specific antibodies (Ab). [d]Immunization and challenge studies were performed in cattle, except that Bd37 was tested in gerbils. [e]+ indicates a positive response; – indicates no response; +/– indicates positive and negative results in separate experiments. [f]Not determined. [g]GenBank accession numbers for proteins not referenced in the paper are: ACS1, AF331454; P0, AF498365; profilin, DQ238092.

*B. bovis, B. bigemina*, or *B. divergens* that contained a number of different antigens were tested (reviewed in (57)). These exoantigens consisted of proteins with molecular masses of 23- and 37–40-kDa (*B. bovis*), 23-, 37–40- and 143-kDa (*B. bigemina*) and 25-, 37-, and 70-kDa (*B. divergens*). The Bd37 protein of *B. divergens* is a potential vaccine candidate for cattle. A fraction of exoantigen containing Bd37 elicited partial protection in cattle (64,65). Using a gerbil infection model, monoclonal antibody (IgG2a isotype) specific for Bd37 was able to confer complete passive protection against homologous Rouen strain and heterologous W8843 strain organisms (66). The 14 amino acid epitope recognized by the antibody had only a single amino acid substitution in the W8843 strain. However, there was minimal protection of gerbils (10% survival rate) when challenged with the 6303E strain, which had five amino acid substitutions in the Bd37 epitope. Recently, a recombinant Bd37 protein vaccine was also able to completely protect gerbils against homologous strain challenge (67). These data, while important for the potential use of Bd37 in cattle, underscore the importance of antigenic *diversity* in developing vaccines against protective antigens, which will be discussed in greater detail below. Interestingly, no homologue of Bd37 has been identified to date in the *B. bovis* genome (http://www.vetmed.wsu.edu/research_vmp/babesia-bovis/index.asp), which is the only cattle *Babesia* genome that has been sequenced. However, this protein, as predicted for the variable merozoite surface antigen (VMSA) family of *B. bovis* and *B. bigemina*, is a glycosylphosphatidyl-inositol (GPI)-anchored membrane protein, which in general may be useful as anti-disease vaccines (57).

### Antibody-proteomic approach

A second approach to identify candidate vaccine antigens was based on identifying merozoite surface proteins and apical complex proteins recognized by bovine immune serum or monoclonal/polyclonal antibodies raised in mice or



Journal compilation © 2006 Blackwell Publishing Ltd, *Parasite Immunology*, **28**, 315–327

rabbits. The rationale for this approach was to target proteins that may be important for erythrocyte invasion by eliciting neutralizing antibodies. Sera from cattle recovered from *B. bovis* or *B. bigemina* infection or mouse monoclonal antibodies identified numerous proteins from surface-labelled merozoites following immunoprecipitation (68–70). Among the *B. bovis* proteins identified as antigenic were 60-kDa RAP-1, 42-kDa merozoite surface antigen-1 (MSA-1), and 44-kDa MSA-2. MSA-1 and MSA-2 are members of the VMSA family described in more detail below. MSA-1 is highly immunodominant, recognized as the major protein band on Western blots by sera from cattle that recovered from infection (71). MSA-1 was an attractive vaccine candidate because it is encoded by a single copy gene, is merozoite surface-exposed, highly antigenic in the native state, and antibodies against native or recombinant MSA-1 neutralized merozoite infectivity *in vitro*, suggesting its importance in merozoite invasion (72–75). However, when put to the test, MSA-1 was not an effective immunogen, as it failed to elicit protective immunity in cattle against homologous strain challenge (73). Wright *et al*. (12) also reported that serologically immunodominant antigens were not protective.

The relevance of antigenic variation of MSA-1 in immune evasion is indicated in recent study comparing *msa-1* genes in Australian K and T vaccine strains with 14 vaccine breakthrough isolates, wherein LeRoith *et al*. found significant genetic variation (76). Predicted amino acid sequence differences as low as 18·7% identity resulted in a complete lack of antibody cross-reactivity between MSA-1 from vaccine strains and their respective breakthrough isolates. Outside of the more conserved amino and carboxy terminal signal sequence regions, only single amino acids or small clusters of amino acids are conserved in all isolates, including a strictly conserved YFK motif at amino acids 174–176.

The MSA-2 proteins are more complex, encoded by a family of four tandem genes in the American isolates (*msa-2a1, msa-2a2, msa-2b*, and *msa-2c*) of which all but *msa2-a2* are expressed as 30–44-kDa proteins and elicit antibody responses upon infection (70,77,78). MSA-2a1 is the original 44-kDa protein described by Jasmer *et al*. (70). The *msa-2* genes are partially conserved among geographically distant strains, but the *msa-2c* gene is the most conserved (77). Bovine antisera specific for MSA-2a1/MSA-2a2, MSA-2b, and MSA-2c significantly blocked attachment and invasion of merozoites to erythrocytes (75), and antibody raised in cattle against recombinant MSA-2c neutralized merozoite infectivity *in vitro* by approximately 50% (79). Using a subset of the same set of vaccine strains and breakthrough isolates as done for MSA-1 analysis, Berens *et al*. found more limited, but still quite significant genetic diversity (as low as 57% identity) among *msa-2* genes (78). Additionally, the Australian *msa-2* locus contained only two genes – *msa-2c* and *msa-2a/b*, the latter closely related to both the *msa-2a* and *msa-2b* genes in American strains of *B. bovis*. MSA-2 contains a strictly conserved YYK motif in approximately the same location as the YFK motif of MSA-1, and has two additional blocks of absolute conservation: VKFCND and SPFM. The VKFCND block is significant, as there is evidence that recombination among VMSA family members occurs (78), and that in some cases the recombination event may be anchored at one or more of the strictly conserved blocks (77,78). Comparison of sequences between VMSA family members has also revealed several other examples where amino acid segments of the hypervariable region (HVR) are highly identical to corresponding sequences in other family members (78). For example, there is a block of amino acids in the HVR of Mexico and Texas MSA-2b that is 92% identical to the corresponding block of amino acids in MSA-1 in four Australian strains. These data are consistent with the possible genetic exchange between *msa-1* and *msa-2* genes.

The HVRs of both MSA-1 and MSA-2 have extremely limited conservation in the carboxy third of the molecules (78, 80). There are no strictly conserved amino acids in this region, and sequence similarity is only 41% among all MSA-1 molecules. As might be predicted by the level of variation, the HVR in MSA-1 is surface-exposed and antibody-accessible, and monoclonal antibodies against this region will inhibit merozoite invasion of erythrocytes, suggesting that the HVR is involved in the invasion process (80).

The extent of genetic polymorphism in VMSA molecules and the ability of antibodies directed against them to inhibit invasion suggests, but does not prove, that the diversity is immunologically selected. Regardless of the mechanisms that select for antigenic variants, antigenic diversity in VMSA proteins is problematic for vaccine development. However, for all MSA-1 and MSA-2 molecules, the predicted overall structure, and presumably the function, appears to be conserved despite sequence polymorphism (76). Coupled with degenerate motifs conserved throughout the family, it may be possible to artificially mimic structure, or to present conserved parasite-associated molecular patterns encoded by these molecules to the host to enhance adaptive or innate responses, respectively. For example, all VMSA family members appear to be GPI-anchored. In other protozoa, including trypanosomes and malarial parasites, GPI anchors are known to activate innate production of proinflammatory cytokines, which can have protective or pathological consequences (81). Recent evidence with *B. divergens* Bd37 shows that inclusion of hydrophobic sequences at the N- or C-terminus of recombinant protein was essential for

generation of effective immunity in vaccinated gerbils (67). Importantly, the C-terminal GPI anchor sequence could be substituted with a non-related sequence derived from the GPI anchor sequence of human delay accelerating factor, suggesting that structure of the protein rather than sequence *per se* was important for inducing protective immunity. Thus, inclusion of naturally occurring hydrophobic amino acid sequences in other membrane protein vaccines may enhance the ability of these molecules to generate a protective response, possibly by acting as a PAMP.

The antibody-based proteomic approach also identified several *B. bovis* proteins, in addition to RAP-1, that are associated with the apical complex organelles. These are the secreted spherical body proteins that include 77-kDa SBP-1 (formerly Bb-1), 225-kDa SBP-2, and 135-kDa SBP-3 (59,82–84). These proteins are each encoded by a single gene, are highly conserved among strains, and localize to both spherical bodies and the cytoplasmic face of the erythrocyte membrane. However, their functions and protective capacity as vaccine antigens have not been determined.

In *B. bigemina,* the notable antigens identified by antibodies directed against the merozoite surface are the RAP-1, gp45, and gp55 proteins (85). In *B. bigemina* RAP-1 is encoded by a polymorphic gene family consisting of transcripts coding for RAP-1a, RAP-1b, and RAP-1c (16,86). However, only RAP-1a could be detected by immunoblotting (87), which suggests that the original 58-kDa protein identified by immune sera is RAP-1a. Native *B. bigemina* RAP-1a protein conferred partial protection, defined by reduction in parasitaemia following challenge with the homologous *B. bigemina* strain (85,88).

Gp45 is a merozoite surface antigen encoded by a single-copy gene and is expressed at the protein level in the Mexico strain of *B. bigemina* (89). However, the gene is not transcribed in the Texcoco strain, and is absent in other American strains examined. Immunization of cattle with native gp45 from the Mexico strain induced partial protection against homologous strain challenge (85), suggesting that this protein may be useful as a component of a vaccine to protect against Mexico strains that do express this surface antigen.

A separate tack was used by Allred and colleagues, who wanted to understand the basis for sequestration of *B. bovis* to microcapillary endothelium (reviewed in (47)). Their studies, using monoclonal antibodies, revealed a doublet of proteins expressed on the surface of infected erythrocytes that changed antigenically over time. These VESA proteins are thought to be encoded by two gene families. The best-characterized family of proteins,VESA1a, is probably encoded by at least 50 genes. Antigenic variation is hypothesized to occur by gene conversion of small segments of *ves1a* into an expression site, although this awaits confirmation. VESA1 proteins were shown to play a functional role in endothelial cell binding *in vitro*, and selection for adhesive or non-adhesive phenotypes revealed differences in VESA1 epitopes (90).

### T cell-proteomic approach

In immunized animals protected from challenge or in persistently infected animals that continually control parasitaemia, antigen-specific $CD4^+$ T cells are believed to be required for the adaptive immune response by producing IFN-γ. In addition to activating macrophages for efficient organism clearance, IFN-γ enhances production of the neutralizing IgG2 antibody (91), which when mixed with IgG1, was shown to passively protect cattle against homologous strain challenge (13). Our laboratory therefore adopted a strategy of using $CD4^+$ T memory cells from cattle that had recovered from *B. bovis* infection and were immune to challenge, to identify potentially important vaccine candidates (reviewed in (16)). Merozoites were solubilized and fractionated by size using continuous flow electrophoresis (CFE), and the resulting ~240 fractions were tested on short-term T cell lines or T cell clones from four animals which had unique MHC class II haplotypes (92,93). Fractions that stimulated high levels of $CD4^+$ T cell proliferation in the majority of cattle were then used to prepare immune sera in rabbits, which in turn was used in expression library screening. This approach identified several known antigens described above that included MSA-1, MSA-2, RAP-1, and SBP-1 (Bb-1) (93), and 12D3 (16). In addition, we identified several novel antigens including heat shock protein (Hsp) 70 and Hsp 90 (94), a 20-kDa Hsp belonging to the α-crystalline protein family which acts to stabilize folding of other proteins (95,96), fatty acyl coenzyme A synthetase (ACS1) that is involved in activation of fatty acyl coA for use in lipid biosynthesis (97), and a phosphoribosomal protein, P0 (Norimine and Brown, unpublished observations), that in other organisms is involved in a complex that interacts with ribosomal RNA and is critical for cell viability (98).

T cell responses to previously known antigens were also characterized. *Babesia bovis* RAP-1, encoded by two nearly identical genes, was expressed and shown to be highly stimulatory for T memory cells (99), as was *B. bigemina* RAP-1a (49,88). Protective immunity in *B. bigemina* RAP-1-immunized cattle correlated with strong $CD4^+$ T cell responses, characterized by high production of IFN-γ (49,88,100), which induced IgG2 by B cells *ex vivo* (51).

*Babesia bovis* RAP-1 was more extensively characterized and shown to contain numerous T cell epitopes recognized by $CD4^+$ T cells from infected and recovered cattle, in both conserved domains and variable repetitive domains



Journal compilation © 2006 Blackwell Publishing Ltd, *Parasite Immunology*, **28**, 315–327

located in the C-terminus (101,102). RAP-1-specific T cells also secreted IFN-γ upon antigen stimulation *ex vivo*. We also had demonstrated T cell proliferative responses to recombinant MSA-1, and the use of MSA-1-specific T cell clones enabled us to demonstrate that epitopes were conserved in Mexico and Texas, but not Australian, strains (103). SBP-1 (Bb-1) also contained T cell epitopes recognized by immune cattle (104,105). In contrast, we were unable to detect T cell responses to MSA-2 proteins (Brown and McElwain, unpublished observations).

Similar immune recognition studies were conducted with the newly identified antigens. Examination of *B. bovis* Hsp20 revealed that *B. bovis* and *B. bigemina* shared at least one epitope that was conserved in all *B. bovis* strains tested, identified by a monospecific mouse serum and a monoclonal antibody (95). However, only one of three post-infection bovine sera recognized a 20-kDa native *B. bovis* protein or recombinant Hsp20 protein on immunoblots, indicating that Hsp20 is not serologically immunodominant during infection. T cell epitopes were also identified in cattle following recovery from infection or immunization with recombinant Hsp20, and one epitope was conserved in *B. bigemina* (106). $CD4^+$ T cells from cattle that recovered from infection or from cattle immunized with recombinant Hsp20 plus IL-12 produced high amounts of IFN-γ *ex vivo* when stimulated with *B. bovis* antigen (95,106).

*Babesia bovis* ACS1 recombinant protein also stimulated T-lymphocyte proliferative responses, but not detectable antibody, from cattle recovered from infection (97). However, the other proteins that were identified by CFE fractionation and expression library serological screening (Hsp70, Hsp90, and P0) did not elicit detectable T-lymphoproliferative responses from recovered cattle and have not been pursued (Norimine and Brown, unpublished observations). This exemplifies a limitation of this strategy, which is the presence of multiple protein fragments in a given fraction that elicited antibody responses in rabbits, but not necessarily T-lymphocyte responses. Another problem is that the approach is time-consuming and not conducive to high-throughput analysis.

Immunization and challenge studies were performed with recombinant *B. bovis* RAP-1 (102) and Hsp20 (Brown *et al*., unpublished data) derived from the Mexico strain and administered separately to cattle. These proteins were selected because of evidence that antibody against RAP-1 blocked sporozoite and merozoite invasion (107,108), the lack of serological immunodominance, the strong recognition by $CD4^+$ T memory cells from protected cattle, and the conservation of epitopes among strains. Protein was administered repeatedly with IL-12 and RIBI adjuvant, until reproducibly strong $CD4^+$ T-lymphocyte proliferation and IFN-γ secretion were observed. All vaccinates mounted strong cell-mediated and IgG1 and IgG2 responses, but none of the cattle were protected against developing disease or parasitaemia following challenge with the virulent $T_2$Bo strain (102; Brown *et al*., unpublished data). Furthermore, high titred immune sera obtained post-immunization failed to neutralize *B. bovis* (Mexico strain) infectivity *in vitro*, in contrast to anti-MSA-1 immune bovine serum (102; Brown *et al.*, unpublished data). RAP-1 amino acid sequences are identical in the $T_2$Bo and Mexico strains, ruling out antigenic variation as an explanation for vaccine failure.

### Genomic approach

Recently, the availability of genomic sequences of *B. bovis* has permitted identification of vaccine candidate antigens by genetic identity with homologous proteins in other protozoa, notably *Plasmodium falciparum.* BLAST analysis was used to identify two genes coding for proteins of interest in expressed sequence tags of the Israel strain of *B. bovis* (109,110). One encodes the AMA-1 homologue of *P. falciparum*, a candidate vaccine antigen that is a micronemal protein expressed on the surface of merozoites, where it becomes processed to smaller soluble fragments during invasion of erythrocytes (111,112). Antibodies against AMA-1 block merozoite invasion in mouse models (113–115). *Babesia bovis* AMA-1 was identified as a low-abundance protein, and a 69-kDa form of the protein is secreted upon invasion of erythrocytes *in vitro* (109). Importantly, rabbit antisera raised against *B. bovis* AMA-1 peptides significantly blocked invasion by *B. bovis in vitro*. BLAST analysis of an expressed sequence tag library was similarly employed to identify a 75-kDa antigen of the apicomplexan thrombospondin-related anonymous protein (TRAP) family (110). TRAP proteins are believed to function in host cell binding, as disruption of the TRAP gene in *P. berghei* negatively affected sporozoite gliding motility, invasion of mosquito salivary glands and infection of hepatocytes (116). *Babesia bovis* merozoites were shown to express TRAP, which localized to the apical end, and was also secreted (110). Antisera raised against TRAP peptides significantly inhibited erythrocyte invasion.

### Combined proteomic-genomic approach

The power of genomics is illustrated in the identification of two putative vaccine antigens, identified by sequence homology as candidate vaccine antigens in related malaria parasites. The *B. bovis* genome of the virulent $T_2$B0 isolate is currently being sequenced and annotated by The Institute for Genomic Research (TIGR), the USDA-ARS Animal Disease Research Unit in Pullman, WA and the WSU Department of Veterinary Microbiology and Pathology.


 

Once the sequence is completed and annotated, the genomic information will be a powerful tool to identify vaccine antigens, selected as in the example above by identity to known protective antigens, or selected by stimulation of a desired immune function. These might include stimulation of memory T cell responses (proliferation and/or IFN-γ secretion) by antigenic fractions, or parasite neutralization by monoclonal antibodies. The proteins identified by these effector T cells or antibodies can be sequenced or subjected to liquid chromatography and tandem mass spectrometry to identify tryptic peptide fragments and the genes that encode them. The advantage of this approach to identification of novel antigens is that proteins can be quickly identified. This approach was recently used to identify a number of novel outer membrane proteins recognized by immune serum IgG from cattle immunized with protective fractions of *Anaplasma marginale* (117,118).

## PROSPECTS FOR VACCINE DEVELOPMENT

Development of effective recombinant vaccines against apicomplexan pathogens, including *Babesia*, *Plasmodium* and *Theileria*, has been frustrating and exceedingly difficult. Since the publication of a previous review article on designing vaccines against *B. bovis* and *B. bigemina* (16) only a handful of new antigens have been identified. Of these, most were identified by genomic analysis (77,109,110). Thus, the completed genome sequences for bovine babesial pathogens should facilitate a more rapid identification of novel potential vaccine antigens, whether a strictly genomic approach or combined genomic and functional proteomic approach is taken. It is likely that a protective subunit vaccine, whether delivered as protein in adjuvant or as a DNA construct, will require inclusion of multiple antigens or immunogenic epitopes of multiple proteins. Vaccine development for cattle is further complicated by the need to protect animals expressing a diversity of MHC class II molecules. Thus, consideration must be given to identifying multiple epitopes that will be recognized by many individuals in a population.

We rationalized that immunization of cattle with a conserved, T cell immunogenic protein such as RAP-1 and appropriate adjuvants to elicit a type 1 immune response should induce protective immunity, but this was not the case (102). Targeting antigens like MSA-1 that are antigenically variant between strains but retain the same structure, presumably to maintain function (76), may prove useful if the function can be disrupted. Similarly, blocking endothelial cell adhesion by immunizing with conserved epitopes in VESA1 or including multiple variants in a vaccine may prevent severe clinical disease. Identification of all *ves* coding sequences in the genome of *B. bovis* will facilitate this latter approach, unless the proposed mechanism of antigenic variation by segmental gene conversion proves true (47).

Progress in vaccine development will also require improved understanding of the mechanisms of immunity in natural hosts of these parasites, so that antigens can be delivered in a way that mimics the innate protective response that can lead to development of protective adaptive immunity. We have hypothesized that control of virulent *B. bovis* infection in naïve animals is largely achieved by activation of the innate immune response in the spleen, whereas protection against clinical disease in persistently infected cattle (concomitant immunity) or vaccinated cattle will rely upon rapid activation of memory and effector $CD4^+$ T cells that secrete IFN-γ, which activates macrophages and provides help for the production of protective antibodies (15,16). Increased understanding of the parasite molecules that activate innate immune responses will help direct the choice of vaccine adjuvants. The induction of a protective IL-12 response in mice to a human apicomplexan pathogen protein, profilin (41), that has no known receptor in humans (45), underscores the importance of studying the immune response to *Babesia* parasites in the natural host.

Finally, development of improved methods for tracking T cells following immunization and challenge will also help determine why vaccine antigens, such as MSA-1 and RAP-1, have failed to provide any protective immunity. MHC class II tetramers loaded with pathogen-derived peptides and labelled with a fluorochrome can be used to stain epitope-specific T cells during the course of immunization or infection (119,120). Not only is the magnitude of the response important, but also the rapidity of the response can be critical, especially with virulent pathogens like *B. bovis* that cause acute disease by 9–10 days post infection. Following the fate of primed $CD4^+$ T cells following parasite challenge will be valuable for understanding the reasons for vaccine failure or success.

## REFERENCES

1 Kjemtrup AM & Conrad PA. Human babesiosis: an emerging tick-borne disease. *Int J Parasitol* 2000; **30**: 1323–1337.
2 Criado-Fornelia A, Martinez-Marcos A, Buling-Saraña A & Barba-Carretero JC. Molecular studies on *Babesia, Theileria,* and *Hepatozoon* in southern Europe. Part II. Phylogenetic analysis and evolutionary history. *Vet Parasitol* 2004; **114**: 173–194.
3 Wright IG & Goodger BV. Pathogenesis of babesiosis. In *Babesiosis of Domestic Animals and Man*, ed. Ristic M. Boca Raton: CRC Press; 1988: 99–118.
4 Clark IA & Jacobsen LS. Do babesiosis and malaria share a common disease process? *Ann Trop Med Parasitol* 1998; **92**: 483–488.
5 Wright IG, Goodger BV & Clark IA. Immunopathophysiology of *Babesia bovis* and *Plasmodium falciparum* infections. *Parasitol Today* 1988; **4**: 214–218.



Journal compilation © 2006 Blackwell Publishing Ltd, *Parasite Immunology*, **28**, 315–327

6 Hemmer RM, Ferrick DA & Conrad PA. Role of T cells and cytokines in fatal and resolving experimental babesiosis: protection in TNFRp55–/– mice infected with the human *Babesia* WA1 parasite. *J Parasitol* 2000; **86**: 736–742.

7 Zintl A, Mulcahy G, Skerrett HE, Taylor SM & Gray JS. *Babesia divergens*, a bovine blood parasite of veterinary and zoonotic importance. *Clin Micro Rev* 2003; **16**: 622–636.

8 Belkaid J, Piccrillo CA, Mendez S, Shevach EM & Sacks DL. $CD4^+CD25^+$ regulatory T cells control *Leishmania major* persistence and immunity. *Nature* 2002; **420**: 502–507.

9 De Vos A & Bock RE. Vaccination against bovine babesiosis. *Ann N Y Acad Sci* 2000; **916**: 540–545.

10 Dagliesh RJ. Babesiosis. In *Immunology and Molecular Biology of Parasitic Infections*, ed. Warren KS. Oxford: Blackwell; 1993: 352–383.

11 Davies SFM, Joyner LP & Kendall SB. Studies on *Babesia divergens* (M'Fadyean & Stockman, 1911). *Ann Trop Med Parasitol* 1958; **52**: 206–215.

12 Wright IG, Casu R, Commins MA *et al.* The development of a recombinant *Babesia* vaccine. *Vet Parasitol* 1992; **44**: 3–13.

13 Mahoney DF. Studies on the protection of cattle against *Babesia bovis* infection. In *The Ruminant Immune System in Health and Disease*, ed. Morrison WI. Cambridge: Cambridge University Press; 1986: 539–545.

14 Edelhofer R, Kanout A, Schuh M & Kutzer E. Improved disease resistance after *Babesia divergens* vaccination. *Parasitol Res* 1998; **84**: 181–187.

15 Goff WL, Johnson CC & Cluff CW. *Babesia bovis* immunity. *In vitro* and *in vivo* evidence for IL-10 regulation of IFN-γ and iNOS. *Ann NY Acad Sci* 1998; **849**: 161–180.

16 Brown WC & Palmer GH. Designing blood stage-vaccines against *Babesia bovis* and *B. bigemina*. *Parasitol Today* 1999; **15**: 275–281.

17 Brown WC. Molecular approaches to elucidating innate and acquired immune responses to the protozoan parasite *Babesia bovis* that causes persistent infection. *Vet Parasitol* 2001; **101**: 233–248.

18 Homer MJ, Aguilar-Delfin I, Telford SR III, Krause PJ, Persing DH. Babesiosis. *Clin Microbiol Rev* 2000; **13**: 451–469.

19 Ruebush MJ & Hanson WL. Transfer of immunity to *Babesia microti* of human origin using T lymphocytes in mice. *Cell Immunol* 1980; **52**: 255–265.

20 Igarashi I, Suzuki R, Waki S *et al.* Roles of $CD4^+$ T cells and gamma interferon in protective immunity against *Babesia microti* infection in mice. *Infect Immun* 1999; **67**: 4143–4148.

21 Clawson ML, Paciorkowski N, Rajan TV *et al.* Cellular immunity, but not gamma interferon, is essential for resolution of *Babesia microti* infection in Balb/c mice. *Infect Immun* 2002; **70**: 5304–5306.

22 Cavacini LA, Parke LA & Weidanz WP. Resolution of acute malaria infections by T cell-dependent non-antibody-mediated mechanisms of immunity. *Infect Immun* 1990; **58**: 2946–2950.

23 Chen D, Copeman DB, Hutchinson GW & Burnell J. Inhibition of growth of cultured *Babesia microti* by serum and macrophages in the presence and absence of T cells. *Parasite Immunol* 2000; **22**: 81–88.

24 Quick RE, Herwaldt BL, Thomford JW *et al.* Babesiosis in Washington State: a new species of *Babesia*? *Ann Int Med* 1993; **119**: 284–290.

25 Aguilar-Delfin I, Homer MJ, Wettstein PJ & Persing DH. Innate resistance to *Babesia* infection is influenced by genetic background and gender. *Infect Immun* 2001; **69**: 7955–7958.

26 East IJ, Zakrzewski H, Gale KR *et al.* Vaccination against *Babesia bovis*: T cells from protected and unprotected animals show different cytokine profiles. *Int J Parasitol* 1997; **27**: 1537–1545.

27 Trueman KF & Blight GW. The effect of age on resistance of cattle to *Babesia bovis*. *Aust Vet J* 1978; **54**: 301–305.

28 Goff WL, Johnson WC, Parish SM, Barrington GM, Tuo W & Valdez RA. The age-related immunity in cattle to *Babesia bovis* infection involves the rapid induction of interleukin-12, interferon-γ, and inducible nitric oxide synthase mRNA expression in the spleen. *Parasite Immunol* 2001; **23**: 463–471.

29 Joyner LP & Donnelly J. The epidemiology of babesial infections. In *Advances in Parasitology*, eds Lumsden WHR, Muller R, Baker JR. London: Academic Press; 1979; 17: 115–140.

30 Christensson DA. Inverse age resistance to experimental *Babesia divergens* infection in cattle. *Acta Vet Scand* 1989; **30**: 453–464.

31 Goff WL, Johnson WC, Horn RH, Barrington GM & Knowles DP. The innate immune response in calves to *Boophilus microplus* tick transmitted *Babesia bovis* involves type-1 cytokine induction and NK-like cells in the spleen. *Parasite Immunol* 2003; **25**: 185–188.

32 Johnson WC, Cluff CW, Goff WL, Wyatt CR. Reactive oxygen and nitrogen intermediates and products from polyamine degradation are babesiacidal *in vitro*. *Ann NY Acad Sci* 1996; **791**: 136–147.

33 Stich RW, Shoda LKM, Dreewes M, Adler B, Jungi TW & Brown WC. Stimulation of nitric oxide production by *Babesia bovis*. *Infect Immun* 1998; **66**: 4130–4136.

34 Shoda LKM, Palmer GH, Florin-Christensen J, Florin-Christensen M, Godson DL & Brown WC. *Babesia bovis*-stimulated macrophages express interleukin-1β, interleukin-12, tumor necrosis factor-alpha, and nitric oxide, and inhibit parasite replication *in vitro*. *Infect Immun* 2000; **68**: 5139–5145.

35 Goff WL, Storset AK, Johnson WC & Brown WC. Bovine splenic NK cells synthesize IFN-γ in response to IL-12 containing supernatants from *Babesia bovis*-exposed monocyte cultures. *Parasite Immunol* 2006; **28**: 221–228.

36 Brown WC, Norimine J, Knowles DP & Goff WL. Immune control of *Babesia bovis* infection. *Vet Parasitol* 2006; **138**: 75–87.

37 Medzhitov R & Janeway CA Jr. Decoding the patterns of self and nonself by the innate immune system. *Science* 2002; **296**: 298–300.

38 Iwasaki A & Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 2004; **5**: 987–995.

39 Takeda K & Akira S. Toll-like receptors in innate immunity. *Int Immunol* 2005; **17**: 1–14.

40 Peng G, Guo Z, Kiniwa Y *et al.* Toll-like receptor 8-mediated reversal of $CD4^+$ T regulatory cell function. *Science* 2005; **309**: 1380–1384.

41 Yarovinski F, Zhang D, Andersen JF *et al.* TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science* 2005; **308**: 1626–1629.

42 Brown WC, Estes DM, Chantler SE, Kegerreis KA & Suarez CE. DNA and a CpG oligonucleotide derived from *Babesia bovis* are mitogenic for B cells. *Infect Immun* 1998; **66**: 5423–5432.

43 Shoda LKM, Kegerreis KA, Suarez CE *et al.* DNA from protozoan parasites *Babesia bovis, Trypanosoma cruzi* and *T. brucei* is mitogenic for B lymphocytes and stimulates macrophage expression of interleukin-12, tumor necrosis factor-alpha, and nitric oxide. *Infect Immun* 2001; **69**: 2162–2171.




 

44 Werling D, Hope JC, Howard CJ & Jungi TW. Differential production of cytokines, reactive oxygen and nitrogen by bovine macrophages and dendritic cells stimulated with Toll-like receptor agonists. *Immunology* 2004; **111**: 41–52.
45 Zhang D, Zhang G, Hayden MS *et al.* A Toll-like receptor that prevents infection by uropathogenic bacteria. *Science* 2004; **303**: 1522–1526.
46 Lauw FN, Caffrey DR & Golenbock DT. Of mice and man: TLR11 (finally) finds profilin. *Trends Immunol* 2005; **26**: 509–511.
47 Allred DR & Al-Khedery B. Antigenic variation and cytoadhesion in *Babesia bovis* and *Plasmodium falciparum:* different logics achieve the same goal. *Mol Biochem Parasitol* 2004; **134**: 27–35.
48 Brown WC & Logan KS. *Babesia bovis*: Bovine helper T cell lines reactive with soluble and membrane antigens of merozoites. *Exp Parasitol* 1992; **74**: 188–199.
49 Rodriguez SD, Palmer GH, McElwain TF *et al.* $CD4^+$ T helper lymphocyte responses against *Babesia bigemina* rhoptry-associated protein-1. *Infect Immun* 1996; **64**: 2079–2087.
50 Brown WC, Woods VM, Dobbelaere DAE & Logan KS. Heterogeneity in cytokine profiles of *Babesia bovis*-specific bovine $CD4^+$ T cell clones activated *in vitro*. *Infect Immun* 1993; **61**: 3273–3281.
51 Brown WC, McElwain TF, Palmer GH, Chantler SE & Estes DM. Bovine $CD4^+$ T-lymphocyte clones specific for rhoptry associated protein-1 (RAP-1) of *Babesia bigemina* stimulate enhanced immunoglobulin G1 (IgG1) and IgG2 synthesis. *Infect Immun* 1999; **67**: 155–164.
52 Estes DM, Closser NM & Allen GK. IFN-γ stimulates $IgG_2$ production from bovine B cells costimulated with anti-μ and mitogen. *Cell Immunol* 1994; **154**: 287–295.
53 McGuire TC, Musoke AJ & Kurtii T. Functional properties of bovine IgG1 and IgG2: interaction with complement, macrophages, neutrophils, and skin. *Immunology* 1979; **38**: 249–256.
54 Gallimore A, Dumrese T, Hengartner H, Zinkernagel RM & Rammensee HG. Protective immunity does not correlate with the hierarchy of virus- specific cytotoxic T cell responses to naturally processed peptides. *J Exp Med* 1998; **187**: 1647–1657.
55 Gallimore A, Hombach J, Dumrese T, Rammensee HG, Zinkernagel RM & Hengartner H. A protective cytotoxic T cell response to a subdominant epitope is influenced by the stability of the MHC class I/peptide complex and the overall spectrum of viral peptides generated within infected cells. *Eur J Immunol* 1998; **28**: 3301–3311.
56 Sher A. Strategies for vaccination against parasites. In *The Biology of Parasitism*, eds Englund PT, Sher A. New York: Alan R. Liss; 1988: 169–172.
57 Schetters TPM & Montenegro-James S. Vaccines against babesiosis using soluble parasite antigens. *Parasitol Today* 1995; **11**: 456–462.
58 Tripp CL, Wagner GG & Rice-Ficht AC. *Babesia bovis*: gene isolation and characterization using a mung bean nuclease-derived expression library. *Exp Parasitol* 1989; **69**: 211–225.
59 Hines SA, Palmer GH, Brown WC *et al.* Genetic and antigenic characterization of *Babesia bovis* merozoite spherical body protein Bb-1. *Mol Biochem Parasitol* 1995; **69**: 149–159.
60 Goodger BV, Waltisbuhl DJ, Commins MA & Wright IG. *Babesia bovis*: dextran sulphate as an adjuvant for and precipitant of protective immunogens. *Int J Parasitol* 1992; **22**: 465–469.
61 Suarez CE, Palmer GH, Jasmer DP, Hines SA, Perryman LE & McElwain TF. Characterization of a gene encoding a 60-kilodalton *Babesia bovis* merozoite protein with conserved and surface exposed epitopes. *Mol Biochem Parasitol* 1991; **46**: 45–52.
62 Harper GS, Hibbs AR, East IJ, Waltisbuhl DJ, Jorgensen WK & Riddles PW. *Babesia bovis*: biosynthesis and localisation of 12D3 antigen in bovine erythrocytes. *Int J Parasitol* 1996; **26**: 1255–1262.
63 Court RA, Sitte K, Opdebeed JP & East IJ. Mapping T cell epitopes of the *Babesia bovis* antigen 12D3: implications for vaccine design. *Parasite Immunol* 1998; **20**: 1–8.
64 Valentin A, Precigout E, L'Hostis M, Carcy B, Gorenflot A & Schrevel J. Cellular and humoral responses induced in cattle by vaccination with *Babesia divergens* culture-derived exoantigens correlate with protection. *Infect Immun* 1993; **61**: 734–741.
65 Carcy B, Precigout E, Valentin A, Gorenflot A & Schrevel J. A 37-kilodalton glycoprotein of *Babesia divergens* is a major component of a protective fraction containing low-molecular-mass culture-derived exoantigens. *Infect Immun* 1995; **63**: 811–817.
66 Precigout E, Delbecq S, Vallet A *et al.* Associations between sequence polymorphism in an epitope of *Babesia divergens* Bd37 exoantigen and protection induced by passive transfer. *Int J Parasitol* 2004; **34**: 585–593.
67 Delbecq S, Hadj-Kaddour K, Randazzo S *et al.* Hydrophobic moieties in recombinant proteins are crucial to generate efficient saponin-based vaccine against apicomplexan *Babesia divergens*. *Vaccine* 2006; **24**: 613–621.
68 McElwain TF, Palmer GH, Goff WL & McGuire TC. Identification of *Babesia bigemina* and *Babesia bovis* merozoite proteins with isolate- and species-common epitopes recognized by antibodies in bovine immune sera. *Infect Immun* 1988; **56**: 1658–1660.
69 Goff WL, Davis WC, Palmer GH *et al.* Identification of *Babesia bovis* merozoite surface antigens by using immune bovine sera and monoclonal antibodies. *Infect Immun* 1988; **56**: 2363–2368.
70 Jasmer DP, Reduker DW, Hines SA, Perryman LE & McGuire TC. Surface epitope localization and gene structure of a *Babesia bovis* 44-kilodalton variable merozoite surface antigen. *Mol Biochem Parasitol* 1992; **55**: 75–84.
71 Brown WC, Logan KS, Wagner GG & Tetzlaff CL. Cell-mediated immune responses to *Babesia bovis* antigens in cattle following infection with tick-derived or cultured parasites. *Infect Immun* 1991; **59**: 2418–2426.
72 Hines SA, Palmer GH, Jasmer DP, McGuire TC & McElwain TF. Neutralization-sensitive merozoite surface antigens of *Babesia bovis* encoded by members of a polymorphic gene family. *Mol Biochem Parasitol* 1992; **55**: 85–94.
73 Hines SA, Palmer GH, Jasmer DP, Goff WL & McElwain TF. Immunization of cattle with recombinant *Babesia bovis* merozoite surface antigen-1. *Infect Immun* 1995; **63**: 349–352.
74 Suarez CE, Florin-Christensen M, Hines SA, Palmer GH, Brown WC & McElwain TF. Characterization of allelic variation in the *Babesia bovis* merozoite surface antigen-1 (*msa-1*) locus and identification of a cross-reactive, inhibition-sensitive MSA-1 epitope. *Infect Immun* 2000; **68**: 6865–6870.
75 Mosqueda J, McElwain TF & Palmer GH. *Babesia bovis* merozoite surface antigen 2 proteins are expressed on the merozoite and sporozoite surface, and specific antibodies inhibit attachment and invasion of erythrocytes. *Infect Immun* 2002; **70**: 6448–6455.
76 LeRoith T, Brayton KA, Molloy JB *et al.* Sequence variation and immunologic cross-reactivity among *Babesia bovis* merozoite



Journal compilation © 2006 Blackwell Publishing Ltd, *Parasite Immunology*, **28**, 315–327

surface antigen 1 proteins from vaccine strains and vaccine breakthrough isolates. *Infect Immun* 2005; **73**: 5388–5394.
77 Florin-Christensen M, Suarez CE, Hines SA, Palmer GH, Brown WC & McElwain TF. The *Babesia bovis* merozoite surface antigen 2 locus contains four tandemly arranged and expressed genes encoding immunologically distinct proteins. *Infect Immun* 2002; **70**: 3566–3575.
78 Berens SJ, Brayton KA, Molloy JB, Bock RE, Lew AE & McElwain TF. Merozoite surface antigen 2 proteins of *Babesia bovis* vaccine breakthrough isolates contain a unique hypervariable region composed of degenerate repeats. *Infect Immun* 2005; **73**: 7180–7189.
79 Willowsky SE, Farber M, Echiade I *et al. Babesia bovis* merozoite surface protein-2c (MSA-2c) contains highly immunogenic, conserved B-cell epitopes that elicit neutralization-sensitive antibodies in cattle. *Mol Biochem Parasitol* 2003; **127**: 133–141.
80 LeRoith T, Berens S, Brayton KA *et al.* The *Babesia bovis* merozoite surface antigen 1 hypervariable region induces surface-reactive antibodies that block invasion. *Infect Immun* 2006; **74**: in press.
81 Gazzinelli RT, Ropert C & Campos MA. Role of the Toll/interleukin-1 receptor signaling pathway in host resistance and pathogenesis during infection with protozoan parasites. *Immunol Rev* 2004; **201**: 9–25.
82 Jasmer DP, Reduker DW, Perryman LE & McGuire TC. A *Babesia bovis* 225-kilodalton protein located on the cytoplasmic side of the erythrocyte membrane has sequence similarity with a region of glycogen phosphorylase. *Mol Biochem Parasitol* 1992; **52**: 263–269.
83 Dowling SC, Perryman LE & Jasmer DP. A *Babesia bovis* 225-kilodalton spherical body protein: localization to the cytoplasmic face of infected erythrocytes after merozoite invasion. *Infect Immun* 1996; **64**: 2618–2626.
84 Ruef BJ, Dowling SC, Conley PG *et al.* A unique *Babesia bovis* spherical body protein is conserved among geographic isolates and localizes to the infected erythrocyte membrane. *Mol Biochem Parasitol* 2000; **105**: 1–12.
85 McElwain TF, Perryman LE, Musoke AJ & McGuire TC. Molecular characterization and immunogenicity of neutralization-sensitive *Babesia bigemina* merozoite surface proteins. *Mol Biochem Parasitol* 1991; **47**: 213–222.
86 Suarez CE, Palmer GH, Hötzel I & McElwain TF. Structure, sequence, and transcriptional analysis of the *Babesia bovis rap-1* multigene locus. *Mol Biochem Parasitol* 1998; **93**: 215–224.
87 Suarez CE, Palmer GH, Florin-Christensen M, Hines SA, Hötzel I & McElwain TF. Organization, transcription, and expression of rhoptry associated protein genes in the *Babesia bigemina rap-1* locus. *Mol Biochem Parasitol* 2003; **127**: 101–112.
88 Brown WC, McElwain TF, Hötzel I, Suarez CE & Palmer GH. Helper T cell epitopes encoded by the *Babesia bigemina rap-1* gene family in the constant and variant domains are conserved among parasite strains. *Infect Immun* 1998; **66**: 1561–1569.
89 Fisher TG, McElwain TF & Palmer GH. Molecular basis for variable expression of merozoite surface antigen gp45 among American isolates of *Babesia bigemina*. *Infect Immun* 2001; **69**: 3782–3790.
90 O'Connor RM & Allred DR. Selection of *Babesia bovis*-infected erythrocytes for adhesion to endothelial cells co-selects for altered variant erythrocyte surface antigen isoforms. *J Immunol* 2000; **164**: 2037–2045.
91 Estes DM & Brown WC. The type 1/type 2 paradigm and regulation of humoral immune responses in cattle. *Vet Immunol Immunopathol* 2002; **90**: 1–10.
92 Brown WC, Logan KS, Zhao S, Bergman DK & Rice-Ficht AC. Identification of *Babesia bovis* merozoite antigens separated by continuous-flow electrophoresis that stimulate proliferation of helper T cell clones derived from *B. bovis*-immune cattle. *Infect Immun* 1995; **63**: 3106–3116.
93 Stich RW, Rice-Ficht AC & Brown WC. *Babesia bovis:* common protein fractions recognized by oligoclonal *B. bovis*-specific $CD4^+$ T cell lines from genetically diverse cattle. *Exp Parasitol* 1999; **91**: 40–51.
94 Ruef BJ, Ward TJ, Oxner CR, Conley PG, Brown WC & Rice-Ficht AC. Phylogenetic analysis with newly characterized *Babesia bovis* hsp70 and hsp90 provides strong support for paraphyly with the piroplasms. *Mol Biochem Parasitol* 2000; **109**: 67–72.
95 Brown WC, Ruef BJ, Norimine J *et al.* A novel 20-kilodalton protein conserved in *Babesia bovis* and *B. bigemina* stimulates memory $CD4^+$ T lymphocyte responses in *B. bovis*-immune cattle. *Mol Biochem Parasitol* 2001; **118**: 97–109.
96 Lee S, Carson K, Rice-Ficht A & Good T. Hsp20, a novel α-crystallin, prevents Aβ fibril formation and toxicity. *Prot Sci* 2005; **14**: 593–601.
97 Norimine J, Ruef BJ, Palmer GH *et al.* A novel 78-kilodalton fatty acyl-CoA synthetase (ACS1) of *Babesia bovis* stimulates memory $CD4^+$ T lymphocyte responses in *B. bovis*-immune cattle. *Mol Biochem Parasitol* 2006; **147**: 20–29.
98 Santos C & Ballesta JPG. Ribosomal protein P0, contrary to phosphoproteins P1 and P2, is required for ribosome activity and *Saccharomyces cerevisiae* viability. *J Biol Chem* 1994; **269**: 15689–15696.
99 Brown WC, McElwain TF, Ruef BJ *et al. Babesia bovis* rhoptry-associated protein-1 is immunodominant for T helper cells of immune cattle and contains T cell epitopes conserved among geographically distant *B. bovis* strains. *Infect Immun* 1996; **64**: 3341–3350.
100 Ruef BJ, Tuo W, Rodriguez SD *et al.* Immunization with *Babesia bigemina* rhoptry-associated protein 1 induces a type 1 cytokine response. Journal of Interferon and Cytokine Research. *J Interferon Cytokine Res* 1997; **17**: 45–54.
101 Norimine J, Suarez CE, McElwain TF, Florin-Christensen M & Brown WC. Immunodominant epitopes in *Babesia bovis* rhoptry-associated protein 1 (RAP-1) that elicit memory $CD4^+$ T lymphocyte responses in *B. bovis*-immune individuals are located in the amino terminal domain. *Infect Immun* 2002; **70**: 2039–2048.
102 Norimine J, Mosqueda J, Suarez C *et al.* Stimulation of T helper cell IFN-gamma and IgG responses specific for *Babesia bovis* rhoptry associated protein 1 (RAP-1) or a RAP-1 protein lacking the carboxy terminal repeat region is insufficient to provide protective immunity against virulent *B. bovis* challenge. *Infect Immun* 2003; **71**: 5021–5032.
103 Brown WC, Palmer GH, McElwain TF, Hines SA & Dobbelaere DAE. *Babesia bovis*: Characterization of the T helper cell response against the 42 kilodalton merozoite surface antigen (MSA-1) in cattle. *Exp Parasitol* 1993; **77**: 97–110.
104 Tetzlaff CL, Rice-Ficht AC, Woods VM & Brown WC. Induction of proliferative responses in T-cells from *Babesia bovis* immune cattle with a recombinant 77 kDa merozoite protein (Bb-1). *Infect Immun* 1992; **60**: 644–652.
105 Brown WC, Zhao S, Woods V *et al.* Identification of two Th1 cell epitopes on the 77 kDa *Babesia bovis* merozoite protein


 

(Bb-1) by use of truncated recombinant fusion proteins. *Infect Immun* 1993; **61**: 236–244.
106 Norimine J, Mosqueda J, Palmer GH, Lewin HA & Brown WC. Conservation of *Babesia bovis* small heat shock protein (Hsp20) among strains and definition of T helper cell epitopes recognized by cattle with diverse major histocompatibility class II haplotypes. *Infect Immun* 2004; **72**: 1096–1106.
107 Mosqueda J, McElwain TF, Stiller D & Palmer GH. *Babesia bovis* merozoite surface antigen 1 and rhoptry-associated protein 1 are expressed in sporozoites, and specific antibodies inhibit sporozoite attachment to erythrocytes. *Infect Immun* 2002; **70**: 1599–1603.
108 Yokoyama N, Suthisak B, Hirata H *et al.* Cellular localization of *Babesia bovis* merozoite rhoptry-associated protein 1 and its erythrocyte-binding activity. *Infect Immun* 2002; **70**: 5822–5826.
109 Gaffar FR, Yatsuda AP, Franssen FF & de Vries E. Erythrocyte invasion by *Babesia bovis* merozoites is inhibited by polyclonal antisera directed against peptides derived from a homologue of *Plasmodium falciparum* apical membrane antigen 1. *Infect Immun* 2004; **72**: 2947–2955.
110 Gaffar FR, Yatsuda AP, Frannsen FF & de Vries E. A *Babesia bovis* merozoite protein with a domain architecture highly similar to the thrombospondin-related anonymous protein (TRAP) present in *Plasmodium* sporozoites. *Mol Biochem Parasitol* 2004; **136**: 25–34.
111 Howell S, Withers-Martinez C, Kocken CH, Thomas AW & Blackman MJ. Proteolytic procession and primary structure of *Plasmodium falciparum* apical membrane antigen-1. *J Biol Chem* 2001; **276**: 31311–31320.
112 Howell SA, Well I, Fleck SL, Kettleborough C, Collins CR & Blackman MJ. A single malaria merozoite serine protease mediates shedding of multiple surface proteins by juxtamembrane cleavage. *J Biol Chem* 2003; **278**: 23890–23898.
113 Anders RF, Crewther PE, Edwards S *et al.* Immunisation with recombinant AMA-1 protects mice against infection with *Plasmodium chabaudi*. *Vaccine* 1998; **16**: 240–247.
114 Kocken CH, Narum DL, Massougbodji A *et al.* Molecular characterization of *Plasmodium reichenowi* apical membrane antigen-1 (AMA-1), comparison with *Plasmodium falciparum* AMA-1, and antibody-mediated inhibition of red cell invasion. *Mol Biochem Parasitol* 2000; **109**: 147–156.
115 Kocken CH, Withers-Martinez C, Dubbeld MA *et al.* High-level expression of the malaria vaccine candidate *Plasmodium falciparum* apical membrane antigen 1 and the induction of antibodies that inhibit erythrocyte invasion. *Infect Immun* 2002; **70**: 4471–4476.
116 Sultan AA, Thathy V, Frevert U *et al.* TRAP is necessary for gliding motility and infectivity of *Plasmodium* sporozoites. *Cell* 1997; **90**: 511–522.
117 Riding G, Hope M, Waltisbuhl D & Willadsen P. Identification of novel protective antigens from *Anaplasma marginale*. *Vaccine* 2003; **21**: 1874–1883.
118 Lopez JE, Siems WF, Brayton KA, Palmer GH, McGuire TC & Brown WC. Identification of novel antigenic proteins in a complex *Anaplasma marginale* outer membrane immunogen by mass spectrometry and genomic mapping. *Infect Immun* 2005; **73**: 8109–8118.
119 Klenerman P, Cerundolo V & Dunbar PR. Tracking T cells with tetramers: new tales from new tools. *Nat Rev Immunol* 2002; **2**: 263–272.
120 Mallone R & Nepom GT. MHC class II tetramers and the pursuit of antigen-specific T cells: define, deviate, delete. *Clin Immunol* 2004; **110**: 232–242.

 